

Mechanical stretch induces podocyte hypertrophy in vitro¹

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Background. Increased intraglomerular pressure is a final pathway toward glomerulosclerosis in systemic hypertension, diabetes, and focal segmental glomerulosclerosis (FSGS). Increased intraglomerular pressure causes stress-tension, or stretch, on resident glomerular cells. However, the effects of stretch on podocyte growth, and the mechanisms that underlie this, have not been elucidated.

Methods. To test the hypothesis that stretch alters podocyte growth, cultured mouse podocytes were exposed to cyclic mechanical stretch created by vacuum; control cells were grown under similar conditions, but not exposed to stretch. Proliferation (cell cycle phases) and hypertrophy (forward light scatter) were measured in stretched and control podocytes by flow cytometry. The role of the cyclin-dependent kinase (CDK) inhibitors, p21 and p27, was examined by stretching podocytes isolated from p21 and p27 knockout (–/–) mice, and the role of specific signaling pathways was assessed by Western blot analysis and blocking studies.

Results. Our results showed that stretch reduced cell cycle progression in wild-type and single p27–/– podocytes and induced hypertrophy in these cells in all phases of the cell cycle at 24, 48, and 72 hours. In contrast, stretch did not induce hypertrophy in single p21–/– and double p21/p27–/– podocytes. Stretch-induced hypertrophy required cell cycle entry, and was prevented by specifically blocking extracellular signal-regulated kinase 1/2 (Erk1/2) or Akt. Although stretch increased p38 activation, inhibition of this pathway had no effect on hypertrophy.

Conclusion. Mechanical stretch induces hypertrophy in podocytes in vitro in all phases of the cell cycle. This effect is cell cycle dependent, and requires p21, Erk1/2, and Akt. Stretch may play a role in podocyte injury when intraglomerular pressure is increased.

Podocytes, also called visceral glomerular epithelial cells, are terminally differentiated cells that line the outer surface of the glomerular basement membrane

[1]. Podocytes prevent protein leakage into the urinary space, and also provide important tensile strength to the glomerular capillaries, by opposing the hydrostatic pressure from within [2]. Podocytes are injured in a variety of immune and nonimmune forms of injury. The responses to podocyte injury include the release of oxidants and proteases, increase in synthesis of growth factors and cytokines, and activation of specific signaling pathways [3]. The consequences of podocyte injury include cell cycle entry with or without proliferation, detachment, accumulation of extracellular matrix proteins, and hypertrophy [4, 5].

In contrast to glomerular endothelial and mesangial cells, podocytes do not typically proliferate. Studies have shown that following podocyte loss, the inability of podocytes to proliferate contributes to the development of glomerulosclerosis [6]. Consequently, in order to cover the denuded glomerular basement membrane, podocytes increase in size due to hypertrophy. Although this is initially compensatory, podocyte hypertrophy is likely maladaptive, and contributes to glomerulosclerosis. Cell growth (proliferation and hypertrophy) has been shown to be cell cycle dependent [7, 8]. Progression through the cell cycle requires that cyclin-dependent kinases (CDKs) are activated by partner cyclins [9]. However, cyclin-CDK complexes are inhibited by CDK inhibitors, which include p21^{Cip1, Waf1} (p21), p27^{Kip1} (p27), and p57^{Kip2} (p57) [10]. Cell growth is also dependent on cell attachment and the presence of mitogens [11], which signal through specific signaling pathways, such as mitogen-activated protein kinase (MAPK) and Akt pathways [12]. Extracellular signal-regulated kinase 1/2 (Erk1/2) and Akt are activated by mitogens, and are proliferative [13, 14]. p38 is activated by environmental stress [15].

Studies have shown that increased intraglomerular capillary pressure causes progressive glomerulosclerosis in diabetic and nondiabetic glomerular disease, and this is associated with podocyte hypertrophy [16]. However, the mechanisms causing podocyte hypertrophy are not known. In order to determine if increased intraglomerular capillary pressure causes podocyte hypertrophy, and to determine the mechanisms underlying this effect, we

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exposed cultured mouse podocytes to cyclic mechanical stretch. Our results show that stress-tension-induced injury generated by mechanical stretch induces podocyte hypertrophy, and this requires the presence of the CDK inhibitor p21. Stretch also reduces podocyte proliferation, due to a decrease in Erk1/2 and Akt.

METHODS

Cell culture

Primary cultures of mouse podocytes were derived from single p21, single p27, double p21/p27 null (−/−) and wild-type (+/+) C57/bl6 mice by differential glomerular sieving as previously described [17]. Briefly, following removal of the kidney capsule, the cortex was finely cut using a scalpel, and gently passed through a sieve with a pore size of 107 μm , placed over a sieve with a pore size of 65 μm . Isolated glomeruli were then passed through a 107 μm sieve a second time, and collected on a sieve with a pore size of 53 μm . Following centrifugation, glomeruli were seeded onto vitrogen-coated 10 cm culture plates (Collagen Biomaterials, Palo Alto, CA, USA) in a media consisting of equal parts conditioned 3T3 media and K1 media (2% NuSerum and 0.5% ITS-Premix) (Becton Dickinson, Bedford, MA, USA), penicillin (100 U/mL), streptomycin (100 $\mu\text{g/mL}$), and glutamine (2 mmol/L) (all from Irvine Scientific, Santa Ana, CA, USA). Nonattached glomeruli were removed by washing after 5 days, at which time adherent glomeruli were transferred to 24-well collagen I-coated plates, using cloning rings. Podocytes were characterized by immunofluorescence for podocin (gift of Peter Mundel, Albert Einstein College of Medicine, New York, NY, USA) and nephrin (gift of Dr. Harry Holthofer; University of Helsinki, Helsinki, Finland), and the absence of α -smooth muscle actin (α -SMA) staining (marker for mesangial cells) and factor VIII immunostaining (marker for endothelial cells). Although all mice were identified by genotyping prior to sacrifice, levels of the CDK inhibitors p21 and p27 were also measured in podocytes *in vitro* by Western blot analysis (see below).

Experimental design

In order to determine the effect of stretch on podocyte proliferation, mouse podocytes were plated onto 6-well, flexible-bottom plates coated with bovine type I collagen (Flexcell International Corp., Hillsborough, NC, USA) at a confluency of about 10% to 20%. Cells were allowed to attach overnight in media containing 5% serum. Podocytes were then synchronized into quiescence by growing cells in medium with 0.5% serum for 72 hours, which prevented apoptosis. To test the hypothesis that stretch induced podocyte hypertrophy, serum-free media was replaced by media containing 5% serum (used as

a source of growth factors). To induce stretch-tension, podocytes were exposed to continuous cycles of mechanical stretch/relaxation using the Flexercell Strain Unit 2000 (Flexcell International Corp.) according to the manufacturer's directions, and as we have previously described [17]. To determine the optimal experimental conditions, podocytes were exposed to either continuous or cyclic mechanical stretch where tensions generated ranged from 5% to 20% biaxial surface elongation.

Based on our preliminary data, we utilized stretch cycles of 0.5 second of stretch and 0.5 second of relaxation for a total of 60 cycles per minute in the current study. The vacuum pressure was −30.92 kPa, which induced an average of 5% circumferential radius elongation in the surface of the culture plates. Control cells were grown under identical conditions, but were not exposed to stretch (i.e., static conditions). Studies of cell growth (proliferation and hypertrophy) were performed in stretched and control cells at 24 hours, 48 hours, and 72 hours after the initiation of stretch (see below). To determine the role of mitogens in stretch-induced hypertrophy, podocytes grown in 0.5% of serum were exposed to stretch in selected experiments. All experiments described were performed a minimum of three times.

Measuring hypertrophy and proliferation

Cell size was used as a measure of hypertrophy, and this was assessed by flow cytometry. Stretched and control (nonstretched) podocytes were harvested by trypsinization, centrifuged, washed twice with phosphate-buffered saline (PBS), and resuspended in 70% ethanol, and stored at −20°C until analysis. Upon thawing, podocytes were stained with propidium iodide (50 $\mu\text{g/mL}$) containing RNase A (30 U/mL) (both from Sigma Chemical Co., St. Louis, MO, USA), and cell size was measured by forward light scatter using flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Cell size was assessed in each phase of the cell cycle. Forward light scatter measures cell diameter, and this measure was used to calculate cell volume for cells in each phase of the cell cycle, using the equation $V = 4/3 \times r^3 \times \pi$. Cell volume is reported in this study.

Proliferation was also determined by measuring the percentage of cells in each phase of the cell cycle at 24 hours in control and stretched cells. All experiments were repeated a minimum of four times. The role of Erk, Akt, and p38 in stretch-induced hypertrophy was determined by stretching podocytes in 0.5% serum and adding specific inhibitors to the media 30 minutes before the initiation of stretch as follows: SB203580 for p38 (20 $\mu\text{mol/L}$) (A.G. Scientific, San Diego, CA, USA), Ly294002 for phosphoinositide 3 kinase (PI₃K) an upstream activator of Akt (50 $\mu\text{mol/L}$) (Cell Signaling Technology, Beverly,

MA, USA), and PD98059 for Erk (50 μ mol/L) (Sigma Chemical Co.).

Western blot analysis

Western blot analysis was used to measure protein levels of specific cell cycle proteins and signaling pathways in stretched and control (nonstretched) cells. Protein was extracted at early (5 minutes, 15 minutes, 30 minutes, and 1 hour), and late (6 hours, 12 hours, 24 hours, 48 hours, and 72 hours) time points following the initiation of stretch using trypsin/collagenase digestion, and lysis in "TG buffer" containing 1% Triton, 10% glycerol, 20 mmol/L Hepes, and 100 mmol/L NaCl with a mixture of protease (Complete Protease Inhibitor) (Boehringer Mannheim Biochemica, Mannheim, Germany) and phosphatase inhibitors [1 mmol/L ethyleneglycol tetraacetic acetate (EGTA), 2.5 mmol/L sodium-pyrophosphate, 1 mmol/L β -glycerophosphate, and 1 mmol/L sodium-orthovanadate] as previously described [18]. Total protein concentration was measured by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. For Western blot analysis, reducing buffer was added to 10 to 20 μ g of protein extract, boiled for 5 minutes and samples were separated on 7.5 or 15% polyacrylamide gel electrophoresis (PAGE) precast gels (BMA, Rockland, ME, USA). Protein was transferred for 1 hour onto PolyScreen polyvinylidene difluoride (PVDF) Transfer Membranes (NEN Life Science Products, Boston, MA, USA); nonspecific background was blocked by washing with 5% nonfat dried milk in Tris-buffered saline with 0.1% Tween (TBST) buffer for 30 minutes, followed by incubation with the primary antibody overnight.

The following primary antibodies were used: p21 (1:200 dilution) (PharMingen, San Diego, CA, USA), Erk1/2-P (Thr202/Tyr204) (1:1000 dilution), Raf-P (Ser259) (1:1000 dilution), Mek1/2-P (Ser217/221) (1:1000 dilution), p90-P (Ser381) (1:1000 dilution), p38-P (Thr180/Tyr182) (1:1000 dilution), Akt-P-S (Ser473) (1:1000 dilution), Akt-P-T (Thr308) (1:1000 dilution), and glycogen synthase kinase (GSK)-3 β -P (Ser9) (1:500 dilution) (all from Cell Signaling Technology, Beverly, MA, USA). All antibodies are specific for the phosphorylated kinases.

After washing with TBST buffer, membranes were incubated with an alkaline phosphatase-conjugated secondary antibody for 1 hour at room temperature. Secondary antibodies used were antimouse IgG (H + L) alkaline phosphatase-conjugated and antirabbit IgG (Fc) alkaline phosphatase-conjugated (all 1:2000 dilution) (Promega, Madison, WI, USA). Protein bands were made visible with the chromagen 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (Sigma Chemical Co.). To ensure that the results obtained by Western blot analysis were not due to unequal protein loading or

protein transfer, we routinely performed double-staining with an antibody to actin (1:4000 dilution) (Boehringer Mannheim) or alpha/beta tubulin (1:2000 dilution) (Neomarkers, Fremont, CA, USA). Total protein was also used as a loading control for the phosphorylated form of specific signaling proteins (Akt, Erk, GSK, p38).

Statistical analysis

Results in this study were expressed as mean \pm SD. Statistical analysis was performed by the Student *t* test.

RESULTS

Mechanical stretch does not induce hypertrophy in the G₀/G₁ phase in the absence of p21

Hypertrophy was measured in stretched and control (nonstretched) podocytes by assessing cell size (volume) utilizing forward light scatter in cells in different phases of the cell cycle utilizing flow cytometry. The results are expressed as the percentage increase in volume in stretched cells compared to non-stretched controls. Figure 1A shows that compared to control (nonstretched) podocytes grown in the presence of serum, stretch increased cell volume in wild-type mouse podocytes in G₀/G₁ at 24 hours ($P < 0.05$ vs. control) and 48 hours ($P < 0.05$ vs. control), but not at 72 hours ($P > 0.05$ vs. control). Stretch also increased cell size in wild-type podocytes in S phase ($P < 0.01$ vs. control), and in G₂/M phase ($P < 0.01$ vs. control) (Fig. 1B and C). Consistent with our previous studies [17], stretch also caused a delay in G₁-phase progression in stretched podocytes grown in serum (Table 1). The data shown are the mean of five separate experiments.

We have previously shown that stretch increases the levels of the CDK inhibitors p21 and p27 in podocytes. To determine the role of p27 in mediating stretch-induced hypertrophy, single p27^{−/−} podocytes were exposed to stretch. Figure 1 shows that compared to nonstretched p27^{−/−} podocytes, cell volume increased in stretched single p27^{−/−} podocytes in G₀/G₁, S, and G₂/M phases, and the increase was statistically significant at all time points ($P < 0.05$ to $P < 0.001$ vs. nonstretched podocytes at 24 hours, 48 hours, and 72 hours). Interestingly, in podocytes lacking p27, the increase in cell size induced by stretch was greater than in wild-type cells exposed to stretch. The data shown is the mean of four separate experiments.

We have previously reported that p21 levels also increase in wild-type podocytes exposed to stretch. In order to determine if p21 also increased in single p27^{−/−} podocytes, we performed Western blot analysis. Figure 2 shows that there was a moderate increase in the expression levels of p21 in stretched p27^{−/−} podocytes. These

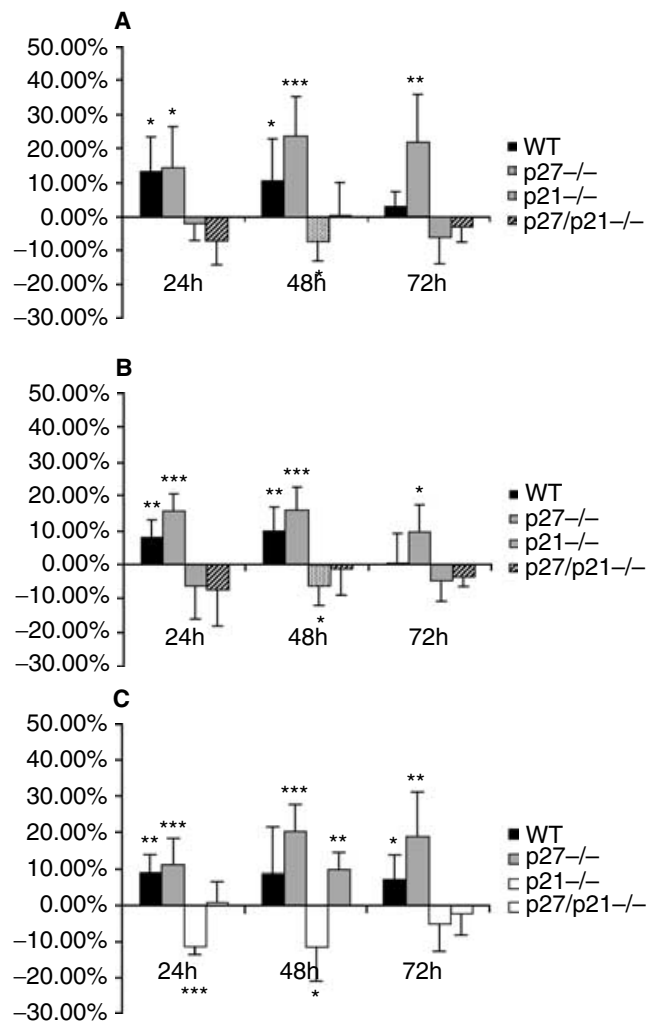


Fig. 1. Stretch increases hypertrophy in wildtype podocytes, but not in cells lacking the cyclin-dependent kinase (CDK) inhibitor p21. Cell volume, a measure of hypertrophy, was measured by fluorescence-activated cell sorter (FACS) analysis in cells in each phase of the cell cycle. The results are expressed as the increase in cell volume of stretched cells compared to nonstretched control cells. (A) Mechanical stretch induces an increase in cell size in wild-type (WT) and p27 null ($-/-$) podocytes in G_0/G_1 . (B) In cells in S phase, stretch induced hypertrophy in wild-type and single p27 $-/-$ podocytes, but not in single p21 $-/-$ and double p21/p27 $-/-$ podocytes. (C) The effect of stretch on hypertrophy was similar in cells in G_2/M phase. * $P < 0.05$ vs. control nonstretched podocytes; ** $P < 0.001$ vs. control nonstretched podocytes; *** $P < 0.001$ vs. control nonstretched podocytes.

results show that p27 alone is not required for stretch-induced hypertrophy.

Single p21 $-/-$ podocytes were utilized to examine the role of this CDK inhibitor in mediating hypertrophy induced by stretch. Figure 1 shows that in contrast to wild-type and single p27 $-/-$ podocytes, stretch did not increase cell size in single p21 $-/-$ podocytes in G_0/G_1 , S, and G_2/M phases at 24 hours, 48 hours, and 72 hours. Indeed, cell size decreased in stretched single

Table 1. Phases of the cell cycle in wild-type, p27 $-/-$, p21 $-/-$, and double p21/p27 $-/-$ podocytes at baseline, in static control cell (C), and stretched (S) cell at 24 hours

	G_0/G_1	S	G_2
Wild-type			
Baseline	83.9% \pm 4.7%	6.3% \pm 3.4%	9.8% \pm 2.7%
C 24 hours	48.5% \pm 7.8% ^a	38.7% \pm 6.1% ^b	12.8% \pm 1.9%
S 24 hours	63% \pm 6.2% ^a	28.5% \pm 6.6% ^b	8.5% \pm 4.8%
p27 $-/-$			
Baseline	79.1% \pm 2.7%	10.7% \pm 3.2%	10.2% \pm 1.8%
C 24 hours	47.9% \pm 3% ^b	7.7% \pm 1.7%	44.4% \pm 1.3% ^a
S 24 hours	60.7% \pm 2.6% ^b	12.3% \pm 2.9%	27% \pm 0.37% ^a
p21 $-/-$			
Baseline	42.8% \pm 7.8%	25.7% \pm 3%	31.5% \pm 5.9%
C 24 hours	22.4% \pm 4.4%	45.7% \pm 9.1%	31.9% \pm 5.8%
S 24 hours	23.6% \pm 0.8%	44.8% \pm 4.6%	31.6% \pm 3.4%
Double p21/p27 $-/-$			
Baseline	46.1% \pm 3.3%	23.2% \pm 6.7%	30.7% \pm 4.5%
C 24 hours	26% \pm 7.2%	44.4% \pm 6.1%	29.6% \pm 3.9%
S 24 hours	27.8% \pm 5.6%	45.9% \pm 5.1%	26.3% \pm 3.1%

^a $P < 0.001$ stretched cells compared to control cells; ^b $P < 0.01$ stretched cells compared to control cells.

p21 $-/-$ podocytes compared to nonstretched control single p21 $-/-$ podocytes. In contrast to wild-type podocytes, stretch did not arrest cells at G_1/S in p21 $-/-$ podocytes (Table 1) ($P > 0.5$ stretched cells compared to control cells). The data shown are the mean of five separate experiments. These results show that the presence of p21 is required for stretch-induced hypertrophy.

We utilized double p21/p27 $-/-$ podocytes to determine if both CDK inhibitors were required for the hypertrophic effect of stretch. Figure 1 shows that stretch did not induce hypertrophy in double p21/p27 $-/-$ podocytes compared to control nonstretched cells in G_0/G_1 , S, and G_2/M phases at 24 hours, 48 hours, and 72 hours ($P > 0.05$ vs. control at each time point). The data shown is the mean of five separate experiments. Stretch did not cause G_1/S arrest in double p21/p27 $-/-$ podocytes (Table 1) ($P > 0.5$ stretched cells compared to control cells). These results show that (1) stretch induces hypertrophy in podocytes, and that this requires the presence of the CDK inhibitor p21, but not p27; (2) stretch also reduces cell cycle progression in single p21 $-/-$ and double p21/p27 $-/-$ podocytes, and (3) that hypertrophy requires that cells arrest at G_1/S .

Stretch-induced hypertrophy requires the presence of mitogens

To determine if stretch-induced hypertrophy requires the presence of serum (a source of growth factors) and cell cycle entry we also measured hypertrophy in stretched podocytes grown in the absence of mitogenic concentrations of serum [0.5% fetal calf serum (FCS)], which is required to prevent apoptosis. Figure 3 shows that in the absence of mitogenic concentrations of serum, stretch does not induce hypertrophy in cells in G_0/G_1 and G_2/M phases at 24 hours and 48 hours. These results show that

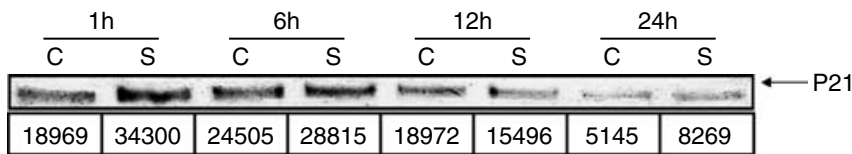


Fig. 2. p21 increases in stretched single p27^{-/-} podocytes. The upper panel shows that the protein levels for p21 were measured by Western blot analysis in single p27^{-/-} podocytes exposed to stretch. The lower panel shows that the densitometric analysis was performed using the housekeeping protein, tubulin, and expressed in arbitrary units. The tubulin blot is identical to the one presented in Figure 5 (same protein isolation). Mechanical stretch increased the levels of p21 compared to control cells not exposed to stretch, and this was maximal at 1 hour. The experiment was performed twice.

cells must engage the cell cycle in order for stretch to induce hypertrophy.

Mechanical stretch induces changes in the levels of Akt and MAPKs

We have previously shown that stretch reduces podocyte proliferation, and in the current study we now show that stretch also induces hypertrophy. To determine which signaling pathways mediate the effect of stretch on podocyte growth, we measured Erk1/2 and Akt. Figures 4 and 5 show that stretch decreased the phosphorylated form of Erk1/2 (Erk1/2 p) in podocytes grown in 5% FCS. Compared to control nonstretched podocytes, this was accompanied by a decrease in the phosphorylated forms of the Erk1/2 regulatory kinases, Raf and Mek (Fig. 4), and also in p90RSK, a kinase downstream of Erk1/2 (Fig. 4).

We also measured the proproliferative kinase, Akt. Stretch decreased the serine and threonine phosphorylated forms of Akt, which are the active forms of Akt (Figs. 4 and 5) in podocytes grown in serum. In contrast to the decrease in ERK and Akt, Figures 4 and 5 show that stretched increased the levels of phosphorylated GSK-3 β kinase. This was accompanied by a marked increase in phosphorylated p38 (Figs. 4 and 5). Finally, stretch did not alter the levels of the H-, K-, and N-RAS isoforms (results not shown).

Inhibiting Erk and Akt decreases stretch-induced hypertrophy

Activation of Erk and Akt is necessary for cell cycle entry and progression through G₁ phase. Figure 6A shows cell cycle synchronization by starving in 0.5% serum for 48h. Figure 6B shows that when control (nonstretched) wild-type podocytes are transferred into 5% serum, cells enter the cell cycle. However, stretch significantly reduces cell cycle progression in cells grown in 5% serum (Fig. 6C). To determine the role of Erk and Akt in mediating hypertrophy induced by stretch, we exposed podocytes to specific inhibitors. Figure 6D shows that when stretch was applied to podocytes grown in serum in the presence of LY294002, an inhibitor of PI₃K an upstream activator of Akt, cells did not engage cell cycle. The binding of PI₃K generated phospholipids to the

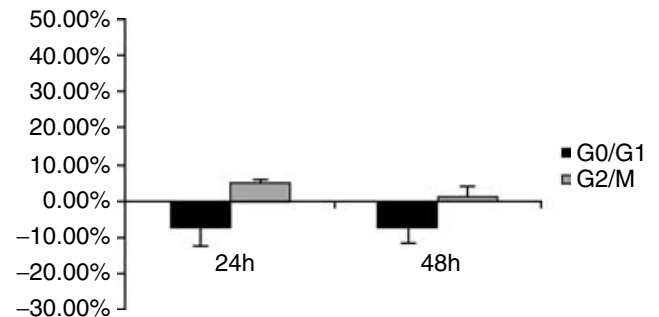


Fig. 3. Stretch does not induce hypertrophy in quiescent wild-type podocytes. Hypertrophy was measured by fluorescence-activated cell sorter (FACS) analysis in cells grown in 0.5% serum, exposed to stretch, and in control cells not exposed to stretch. Stretch did not induce a statistically significant increase in cell size in wild-type podocytes in G₀/G₁ phase, nor in G₂/M, compared to nonstretched cells. $P > 0.05$ control cells compared to stretch cells.

pleckstrin homology (PH) domain of Akt leads to the translocation of Akt to the membrane and induces conformational changes that are required for proper phosphorylation and activation of Akt. As no specific inhibitor of Akt is available an indirect blocking method by using LY294002 was chosen. Similarly, exposing podocytes to PD98059, the Erk1/2 inhibitor (Fig. 6E), also prevented cell cycle entry. The cell cycle distribution and the total cellular events measured are presented in Table 2.

To determine if inhibiting cell cycle entry prevented stretch-induced hypertrophy, cell size was measured in stretched cells exposed to Erk and Akt inhibitors. Figure 6F shows that hypertrophy induced by stretch was significantly reduced in cells in G₀/G₁. In contrast, inhibiting Erk and Akt did not significantly alter cell size in cells in G₂/M. These results show that stretch did not induce hypertrophy in cells that were unable to enter cell cycle, and also shows that the effects of serum are mediated by Erk1/2 and/or Akt for stretch-induced hypertrophy.

p38 has been shown to cause hypertrophy in cardiac cells [19], and our results showed that p38 phosphorylation was increased in podocytes exposed to stretch (Figs. 4 and 5). In contrast to inhibiting Erk or Akt, our results showed that inhibiting p38 did not affect stretch-induced hypertrophy in podocytes (results not shown). Moreover, p38 phosphorylation coincided with an increase in

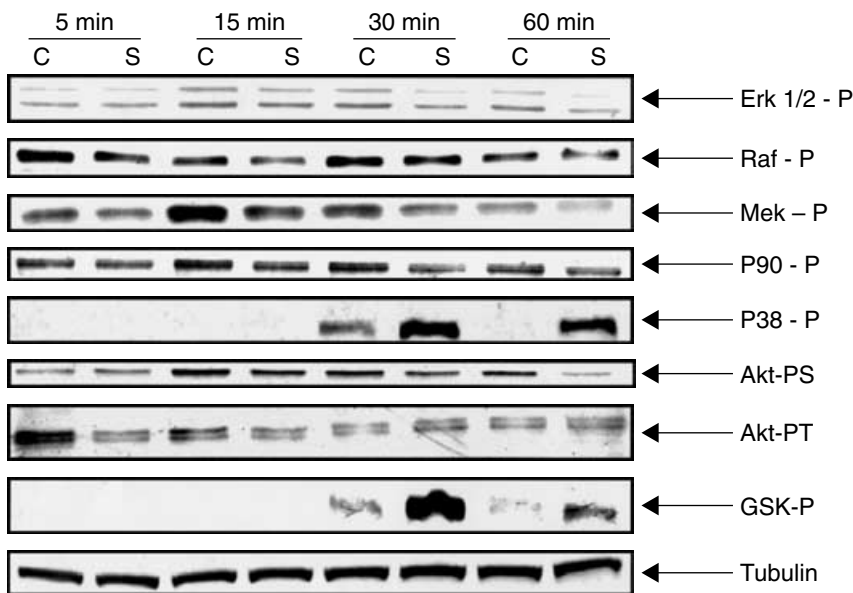


Fig. 4. Differential expression of signaling proteins in control nonstretched and stretched podocytes grown in the presence of 5% serum. Control (C) and stretched (S) podocytes were grown in 5% serum, a source of growth factors. Stretch reduced the levels of active extracellular signal-regulated kinase 1/2 (Erk1/2-P), Raf (Raf-P), and mitogen-activated protein kinase kinase (MEK) (MEK-P). Stretch increased phosphorylated p38 (p38-P) and GSK-3 β (GSK-3 β -P). In contrast, the increase in the serine (Akt-PS) and threonine (Akt-PT) phosphorylated forms of Akt induced by serum in control cells was reduced by stretch. The housekeeping protein, tubulin, was used as a protein-loading control.

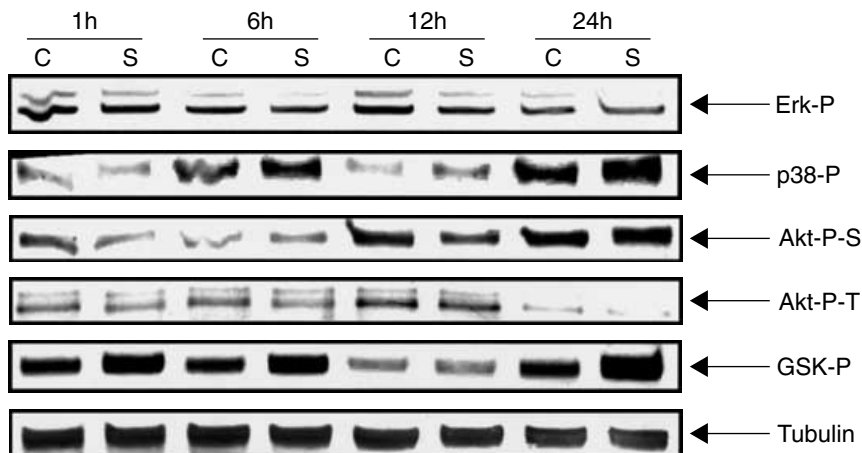


Fig. 5. Western blot analysis of protein isolate from control and stretched wild-type podocytes at later time points. Mechanical stretch reduced phosphorylated extracellular signal-regulated kinase (Erk) and Akt (Akt-PS, Akt-PT), and increased the phosphorylated levels of p38 (p38-P) and GSK-3 β (GSK-3 β -P).

GSK-3 β phosphorylation. Inhibiting p38 phosphorylation with SB203580 also decreased the phosphorylation of GSK-3 β (Fig. 7). These results show that p38 is upstream of GSK-3 β , but inhibiting the phosphorylated form that increases upon stretch, has no effect on hypertrophy.

DISCUSSION

The growth response to podocyte injury is a critical determinant in the course and prognosis of specific glomerular diseases. Typical podocyte growth responses to injury include a lack of proliferation and an increase in cell size due to hypertrophy. However, the mechanisms governing podocyte growth in states of increased intraglomerular pressure, including diabetic nephropathy, are not known. Our results show that stress-tension induced by mechanical stretch induces podocyte hypertrophy, and this re-

quires the CDK inhibitor p21, and the signal proteins Erk1/2 and Akt.

Increased intraglomerular pressure characterizes progressive glomerulopathies, and studies have shown that normalizing intraglomerular pressure reduces progressive glomerulosclerosis [20]. Although podocytes are injured by increased intraglomerular pressure, little is known about the mechanisms underlying this effect. In an attempt to simulate these events in vitro, cultured mouse podocytes were exposed to stretch as we [17] and others [21] have described, similar to what other investigators have done for mesangial [22] and vascular smooth muscle cells [23].

The first major finding in this study was that stretch induced hypertrophy in podocytes in culture. This occurred only when podocytes were grown in the presence of serum, used as a source of growth factors, and not in quiescent (serum-free) podocytes. Inhibiting entry

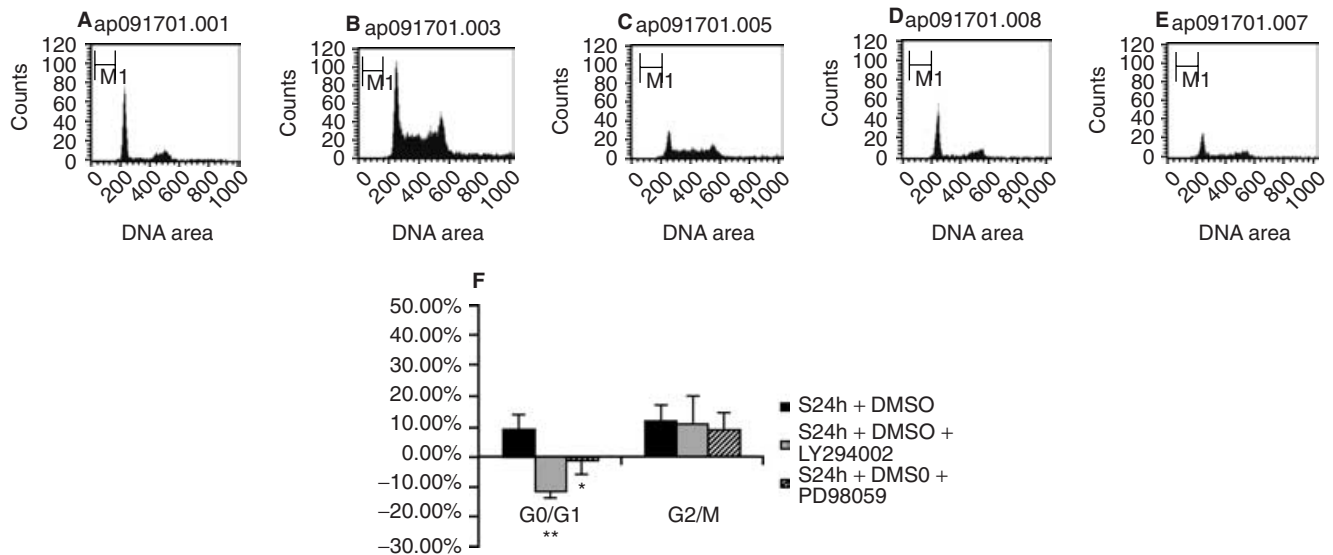


Fig. 6. Extracellular signal-regulated kinase (Erk) and Akt are required for cell cycle progression. Phases of the cell cycle were measured by fluorescence-activated cell sorter (FACS) analysis. (A) Wild-type podocytes are grown in 0.5% serum synchronized growth-arrested cells. (B) Growing nonstretched control podocytes in 5% serum caused cycle entry and progression. (C) Stretch reduced cell cycle progression. (D) The addition of Ly294002, an inhibitor of Akt, to podocytes grown in serum, prevented cell cycle entry. (E) PD98059, an Erk inhibitor, prevented entrance into the cell cycle. (F) When grown in 5% serum and exposed to stretch, Akt and Erk inhibitors reduced stretch-induced hypertrophy in cells in G₀/G₁, but had no major effect on cells in G₂/M. ***P* < 0.01 inhibitor-treated cells compared to untreated cells; **P* < 0.05 inhibitor-treated cells compared to untreated cells.

Table 2. Cell cycle distribution of the data presented in Figure 6.

	D0 + DMSO	C 24 hours + DMSO	S 24 hours + DMSO	S 24 hours + DMSO + LY294002	S 24 hours + DMSO + PD98059
G ₀ /G ₁	66.9%	23.8%	36.6%	62.8% ^a	52.1% ^a
S	14.4%	59.7%	48.7%	13.6% ^a	22.1% ^a
G ₂ /M	18.7%	16.5%	14.7%	23.6%	25.8%
Total events	2210	9703	2919	1822	1618

DMSO is dimethylsulfoxide.

This experiment was performed a total of three times.

^a*P* < 0.01 compared to S 24 hours + DMSO.

into cell cycle by suppressing the proliferative kinases, Erk1/2 and Akt, abolished stretch-induced hypertrophy. Thus, cell cycle entry is required for hypertrophy. Our results also showed that the effects of stretch on hypertrophy were not limited to cells in a specific phase of cell cycle, because cell size increased in podocytes that were in G₀/G₁, S, and G₂/M phases. Previous studies have also shown that stretch induces hypertrophy in skeletal muscle cells and cardiac myocytes [24, 25]. In this study, we assessed hypertrophy by measuring changes in cell size by fluorescence-activated cell sorter (FACS) analysis, because we and others have shown that FACS analysis is a very sensitive and specific method for measuring cell hypertrophy [26, 27]. Moreover, a major advantage of FACS over other measures of hypertrophy such as protein:DNA ratios, is the ability to measure hypertrophy in each phase of the cell cycle. Endlich et al [21] had originally described a decrease in cell size by mechanical stretch. They were looking for changes in the area of the cell body by estimating the product of cell body width and length. The definition of hypertrophy is an in-

crease in cell size/volume/protein. As the cell is a three-dimensional structure, the product of body width and length neglects the third important parameter (i.e., cell height). For this reason FACS might be a more sensitive approach for looking for changes in cell size than morphometry. Preliminary data in mesangial cells (not shown) also showed that protein:DNA measurements, and the results obtained by FACS, were similar. We therefore have reported our FACS data.

We and others have recently shown a role for the CDK-inhibitors p21 and p27 in renal cell growth [26, 28, 29]. A second major finding in this study was that cell size did not increase in single p21^{-/-} and double p21/p27^{-/-} podocytes exposed to stretch. In contrast, hypertrophy was detected in wild-type and single p27^{-/-} podocytes exposed to stretch. We also asked if the induction of hypertrophy by stretch required cell cycle arrest. Our results showed that stretch delayed cell cycle progression in wild-type and single p27^{-/-} podocytes grown in serum (a source of mitogens). However, stretch did not arrest p21^{-/-} and double p21/p27^{-/-} cells exposed to serum.

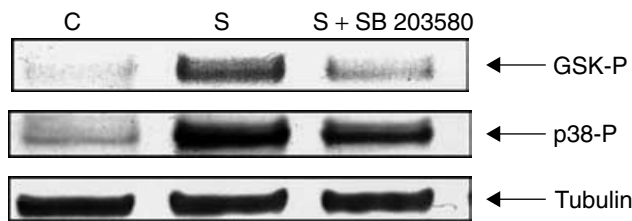


Fig. 7. Inhibiting p38 reduced GSK-3 β phosphorylation. Stretch (S) (lane 2) increased phospho-p38 compared to control cells (C) (lane 1). The increase coincided with an increase in GSK- β . Lane 3 shows that inhibiting p38 with SB203580 reduced the levels of phosphorylated p38 and GSK-3 β in stretched cells.

Taken together, these results show that p21 is required for stretch-induced hypertrophy in podocytes, and that hypertrophy only occurs in cells that enter the cell cycle, but are capable of arresting. Furthermore, cell cycle arrest and hypertrophy requires the presence of the CDK inhibitor p21. The antiproliferative effect of stretch on podocytes differs from the proproliferative effect of stretch on mesangial and vascular smooth muscle cells [22, 23]. Surprisingly, cell types lacking p21 showed a tendency to cell size decrease by stretch. The reason for this is unclear. As our data show no difference in cell cycle progression between control and stretched cells we cannot argue that the presence of p27 pushes cells in the cell cycle and therefore reduces cell size. One could only speculate that the lack of p21 makes a cell more vulnerable to stretch and therefore induces cell size decrease.

A role for specific CDK inhibitors has been shown in renal growth. p21 is necessary for glomerular hypertrophy accompanying experimental diabetic nephropathy [30] and glomerular hypertension [28]. Overexpressing p21 induces tubular cell hypertrophy [31], and the upregulation of p21 by heme oxygenase-1 underlies hypertrophy in LLC-PK₁ cells [32]. Interestingly, our study showed that p21 levels increased early in p27^{-/-} podocytes exposed to stretch, and was therefore likely sufficient to overcome the loss of p27. The CDK inhibitor p27 is also a critical determinant of mesangial cell hypertrophy induced by hyperglycemia, and overexpressing p27 alone is sufficient to induce renal cell hypertrophy [29]. A role for p27 in angiotensin II-mediated hypertrophy of proximal tubular cells [33] and vascular smooth muscle cells [34] has also been reported. Finally, we have recently shown that both p21 and p27 are required for maximal mesangial cell hypertrophy induced by TGF- β [35]. However, the results of the current study show that both CDK inhibitors are not required for maximal stretch-induced hypertrophy, because cell size was similar in p21^{-/-} and double p21/p27^{-/-} podocytes exposed to stretch. Taken together, the results of the current study indicate that the role of specific CDK inhibitors in hypertrophy may be dependent on the cell type, and also on the type of injury.

Specific signaling pathways govern proliferation in response to extracellular cues, which in turn modulate specific cell cycle regulatory proteins [13, 14]. Erk1/2 has been shown to increase cyclin D1 transcription and to modify other cell cycle proteins [36]. This contrasts to the Erk1/2 in mesangial cells exposed to stretch [37]. A third finding in the current was that stretch significantly reduced the Erk1/2 pathway in podocytes grown in the presence of mitogens (serum). Our results also showed that stretch decreased the active forms of Raf and Mek1/2, activators of Erk1/2. Similarly, stretch also decreased phosphorylated p90RSK, which is downstream of Erk1/2.

Akt is a potent proproliferative signaling protein, and induces proliferation by increasing cyclin D1 transcription and translation [37, 38] and by inhibiting the CDK inhibitors p21 and p27 [39, 40]. Our results showed that as expected, Akt was increased in control podocytes grown in serum, not exposed to stretch. However, stretch significantly reduced both forms of phosphorylated (and active) Akt.

The decrease in Erk and Akt levels suggested a potential role for these pathways in mediating the effects of stretch on podocyte growth. Thus, to demonstrate the role of Erk and Akt in stretch-induced hypertrophy, we inhibited these pathways pharmacologically in podocytes grown in serum, prior to the application of stretch. These kinase inhibitors have been described in the literature as highly specific at the concentrations used in our studies [41]. Of course, crossreactions with other, possibly unknown, kinases can not be excluded. For the selection of the inhibitor concentrations we used several different approaches. First, we contacted the providers for recommendation. Second, we studied the literature for used concentrations in different cell lines [41]. Third, we performed some pilot experiments in our laboratory using kinase inhibitors at different concentrations in mesangial cells. Our results showed that by inhibiting Erk and Akt, the hypertrophic effects of stretch in wild-type and single p27^{-/-} podocytes were abolished. These results show that Erk and Akt have a role in stretch-induced hypertrophy in podocytes.

We also studied p38 MAPK in stretched podocytes because this pathway is increased in response to certain cell stressors and has been shown to cause hypertrophy in cardiac myocytes [19]. Our results showed that stretch caused a marked increase in p38 phosphorylation. However, inhibiting p38 with different concentrations of SB203580 had no effect on podocyte hypertrophy or proliferation. Thus, the functional role of increased p38 in stretched podocytes remains to be determined.

Although GSK-3 β was initially thought to be restricted to glycogen metabolism, studies have shown that it is also a critical signaling protein. Activation by dephosphorylation induces apoptosis. A recent study also showed that inactivation of GSK-3 β by phosphorylation is required

for cardiomyocytes to undergo hypertrophy [42]. GSK-3 β is negatively regulated by phosphorylation by Akt [43]. Thus, by phosphorylating and inhibiting GSK-3 β , Akt also increases cell number by inhibiting apoptosis [44]. Although we anticipated that the decrease in Akt caused by stretch would lead to activation (dephosphorylation) of GSK-3 β , our results showed that stretch increased GSK-3 β phosphorylation, and therefore inactivation. However, our results showed that inhibiting p38 decreased GSK-3 β phosphorylation, suggesting that p38 might regulate GSK-3 β in stretched podocytes. Moreover, the lack of podocyte apoptosis in stretched cells may be due to GSK-3 β inactivation.

CONCLUSION

We show that mechanical stretch induces hypertrophy in cultured podocytes. This requires entry into cell cycle, and is dependent on the presence of the CDK inhibitor p21, but not p27. These data provide a potential mechanism for the increase in podocyte size in diseases associated with increased intraglomerular pressure.

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